AMINO ACID ANALYSIS: HYDROLYSIS, COLOR REAGENT, AND SENSITIVITY

by

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I wish to thank my wife Patsy for her love, understanding, and tremendous help during this time period.

I also wish to thank Dr. Lynn S. Bates for his guidance.

INTRODUCTION

Automated ion-exchange chromatography is today the most widely used method for amino acid determinations. There have been improvements in resin technology and instrumentation, but research is still needed to improve current techniques.

One major detection problem is proline quantification. Proline, an imino acid, reacts with ninhydrin to yield a yellow colored compound instead of the purple complex that is formed by the reaction of the α-amino acids. The yellow color has an absorbance maximum that is different from the purple complex. One objective of this study was to develop a color reagent that will allow detection of both imino and α-amino acids at one wavelength.

There is increasing demand for more sensitive detection of amino acids. Analysis of very low levels of amino acids is of particular importance in specialized research where amino acids and proteins occur in low concentrations. A portion of this study was devoted to the development of methods to optimize color yield and thereby increase analyzer sensitivity.

Another important step in amino acid analysis is hydrolysis. Hydrolysis with HCl is the most common method used. Alternate hydrolysis methods need to be investigated for routine analysis of food and feed samples. The third portion of this study was timed hydrolysis of a cereal product to compare HCl and p-toluenesulfonic acid hydrolysates efficacy and amino acid destruction.

LITERATURE REVIEW

Color Reagents for Amino Acid Analysis

The detection of amino acids is most commonly done with ninhydrin (2,2-dihydroxy-1,3-indandione). Ninhydrin decarboxylates and deaminates the α-amino acids in stoichiometric amounts, to form carbon dioxide and an aldehyde with one less carbon than the α-amino acid. Ammonia, hydrindantin, and Rhuhemann's Purple (diketohydrindylidene-diketohydrindamine) are produced from this reaction in variable amounts. Rhuhemann in 1910 and 1911 discovered the reaction of ninhydrin with amino acids and proposed a possible reaction mechanism (McCaldin, 1960). Since that time many different mechanisms have been postulated. The currently most accepted mechanism is given in Fig. 1. (Bottom et al., 1978). Ninhydrin (1) is shown to tautomerize to 1,2,3-indantrione (2) which forms a Schiff's base with the amino acid. The ketimine formed (3) decarboxylates, yielding an aldehyde and the intermediate 2-amino-1,3-indandione (4). Condensation of this intermediate with another molecule of ninhydrin forms Rhuhemann's Purple. There is still controversy as to the exact role of hydrindantin (reduced ninhydrin) in the reaction mechanism (Lamothe and McCormick, 1973).

Moore and Stein (1948) were the first to adapt the ninhydrin reaction to quantitative determination of amino acids. In all previous work the color development had been carried out in tubes exposed to air. They felt that dissolved oxygen present in the reagents was responsible for the non-linear yields. By developing the color in tubes evacuated to 20 mm the relationship of color yield to amino acid concentration was found to be more linear. They experimented with adding either hydrindantin or a strong reducing agent to the reaction medium. Both were found to completely block the oxidative side reaction and give reproducible linear results. At that

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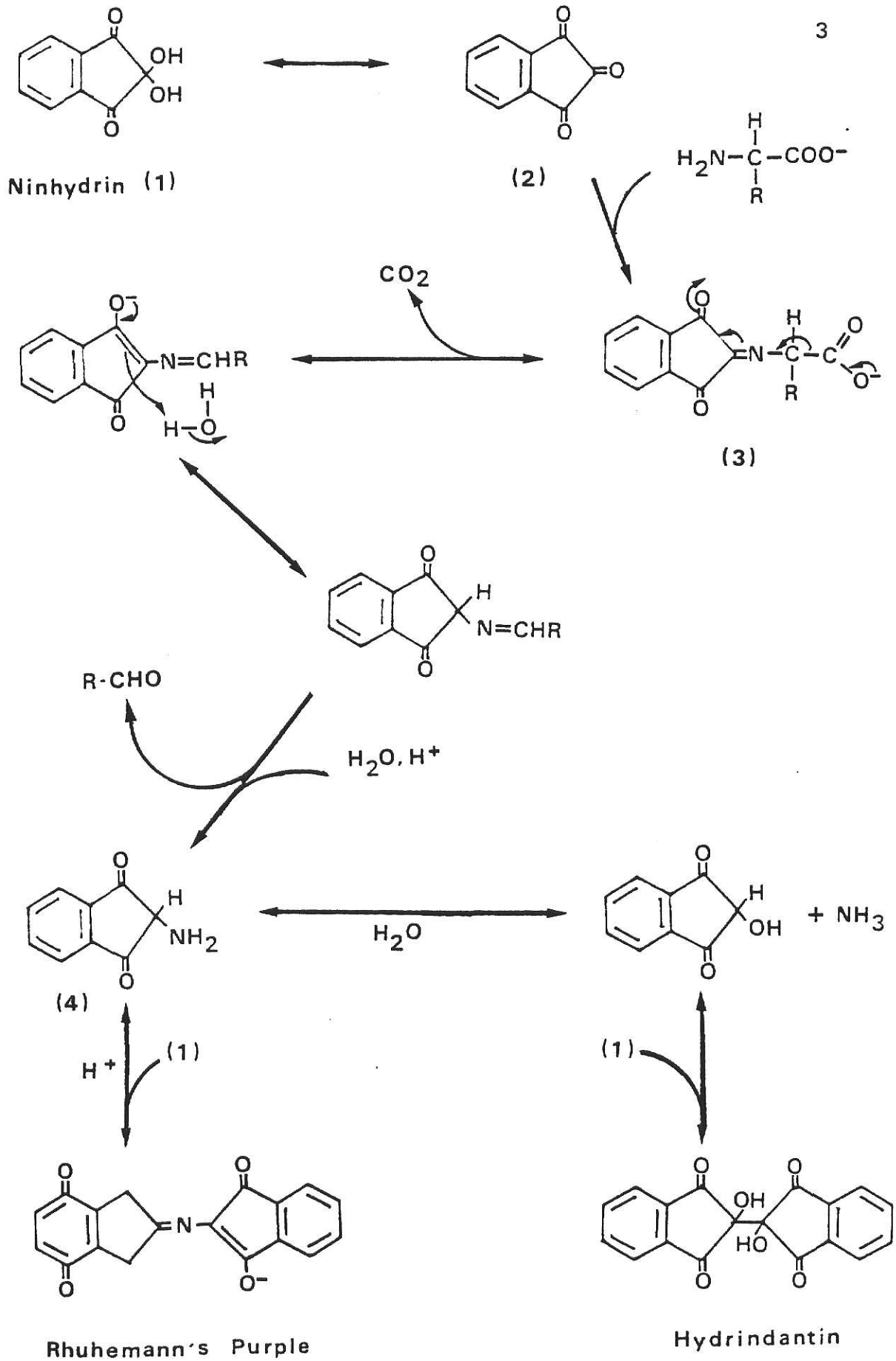

Fig. 1. Mechanism of the Ninhydrin Reaction

time there was not a source of crystalline hydrindantin so stannous chloride was added to the reaction medium to reduce ninhydrin to hydrindantin in situ.

Later in 1954, Moore and Stein returned to adding hydrindantin directly to eliminate the precipitation of tin salts. They also changed from a sodium citrate to a sodium acetate buffer to increase buffering capacity. The concentration of methyl cellosolve (the monomethyl ether of ethylene glycol) was increased to prevent hydrindantin precipitation. This reagent (2% ninhydrin, 0.3% hydrindantin in 3:1 methyl cellosolve and 0.4 M pH 5.5 sodium acetate buffer) when reacted with amino acids at 100°C yields a purple complex, absorbance maximum (Amax) 570 nm, with α-amino acids and a yellow complex, Amax 440 nm, with imino acids. Each amino acid produces a different amount of color per mole, therefore a separate color factor was used for each amino acid to obtain quantitative values.

Spackman, Stein and Moore (1958) developed the first automatic amino acid analyzer. Their 1954 fraction collector ninhydrin reagent was adapted for use in this instrument. Hydrindantin tended to precipitate at the 25% methyl cellosolve level that resulted when 2 parts effluent were mixed with 1 part ninhydrin reagent. The decreased level of oxygen in the automated system allowed hydrindantin to be used at 0.55 g per liter which eliminated precipitation, but the reagent was stable for only two weeks. A more stable reagent was formed by replacing hydrindantin with stannous chloride. At 0.4 g stannous chloride dihydrate per liter the reagent could be stored for one month without deterioration.

This original reagent is currently being used in many automatic amino acid analyzers. The precipitation problem with stannous chloride described by Moore and Stein (1954) is still present. Other reducing agents such as titanous chloride (James, 1971 and 1978) and sodium borohydride (Takahashi, 1978) have been used to replace stannous chloride. Both report equal or

better stability and color yield without the precipitation problem. Other methods have proposed adding the reductant to the eluent buffers (Knight, 1968, Niece, 1974, and Schwerdtfeger, 1962). An air stable ninhydrin reagent can then be used that is freshly reduced when mixed with column effluent. Kirschembaum (1965) substituted a portion of dimethyl sulfoxide (DMSO) for methyl cellosolve and reported very little precipitation with a 60% methyl cellosolve and 40% DMSO mixture. Moore (1968) completely replaced methyl cellosolve with DMSO and stannous chloride with hydrindantin. In order to dissolve all reagent constituents, he changed the sodium acetate buffer to a 4 M pH 5.2 lithium acetate buffer. Hydrindantin is 1.5 times more soluble in DMSO than in methyl cellosolve therefore precipitation is eliminated. DMSO does not contain peroxides which are present in methyl cellosolve. Lack of peroxides gives an increase in stability and color yield. Moore suggested the teflon tubing, which is permeable to oxygen be replaced with saran tubing.

Lamothe and McCormick (1972) studied the optimum pH value for the ninhydrin reaction. They found a pH of 5.0 to 5.2 produced optimal color yields in either methyl cellosolve or DMSO. Friedman and Williams (1974) looked at the stability of Rhuhemann's Purple (RP) under various conditions. They found RP to be stable in the pH range of 7.0 to 14.0 in methyl cellosolve, but RP is destroyed in the acid pH range by acid hydrolysis. When DMSO was substituted for methyl cellosolve, RP was found to be 10 times more stable in the acid pH range.

To further increase sensitivity in amino acid analysis the production of fluorescent compounds using fluorescamine and o-phthalaldehyde has been used for fluorescent amino acid quantification (Undenfriend et al., 1972 and Roth, 1971 and Roth and Hampai, 1973). Fluorescamine reacts with primary

amines at an alkaline pH and must be made and stored in acetone since it is unstable in water. o-Phthalaldehyde is stable in water but requires an alkaline pH and the presence of 2-mercaptoethanol for fluorescence development. Benson and Hare (1975) compared the sensitivity of ninhydrin, fluorescamine, and o-phthalaldehyde. They reported ninhydrin and fluorescamine to be equal in sensitivity, but o-phthalaldehyde to be 5 to 10 times more sensitive. However, these two compounds react only with primary amines and not imino acids such as proline.

Ninhydrin, fluorescamine, and o-phthalaldehyde are not good color reagents for proline detection. There is a need for a color reagent that will allow detection of proline at the same wavelength as the other amino acids. A possible color reagent is isatin. Isatin has been used in quantitative determination of proline by paper chromatography (Barrollier et. al., 1956 and Hrabětová and Tupý, 1960). The reagent used by Barrollier and his associates produced a color complex that had an Amax at 610 nm and followed Beer's law. In their study, proline was analyzed in a range of 5 to 20 ug with an accuracy of $\pm$ 2.5%. Isatin has promise because its Amax is very close to the 570 nm Amax of ninhydrin. This would allow a simple colorimeter to be used if a mixed reagent of ninhydrin and isatin were developed and their absorbance maxima peaks overlapped.

Sensitivity

Increased sensitivity in amino acid analysis has been achieved by reducing resin bed cross-section area and resin bead diameter, and by increasing the optical path length of the flowcell (Hare, 1977). There has also been experimentation with increasing the reaction bath temperature. The ninhydrin reaction is carried out in a post-column, teflon tube

reaction coil immersed in boiling water. The maximum color develops in 8 to 15 minutes at 95 to 100°C in open-ended coils (Spackman, Stein, and Moore, 1958). Anderson and his associates (1968) found that if the reaction coil is kept under high pressure the temperature can be raised to 135°C and maximum color development will occur in 1.3 minutes. A shorter color development time allows the use of a shorter reaction coil which will reduce post column mixing and peak broadening and in that way increase sensitivity.

Jonker and associates (1978) compared the color yield to reaction temperature with a reaction time of one minute (Fig. 2). The results show a maximum color yield at 140 to 150°C for the three amino acids used. This is consistant with findings by Anderson, et al. (1968). Conducting the reaction at 140°C will allow quantification at the maximum portion of the color yield curve instead of on the ascending portion. Jonker and his co-workers used a very elaborate system utilizing an air-thermostat of a liquid chromatograph (Perkin Elmer 1220) to control the temperature between 100° and 150°C.

Hydrolysis

With the increased precision in amino acid analysis the limiting factor in the determination of the composition of a protein is the extent to which the composition of the hydrolysate accurately represents the amino acid composition of the parent protein. A truly representative hydrolysate is difficult to obtain in practice due to the degradation of the labile amino acids while liberating the more slowly released amino acids.

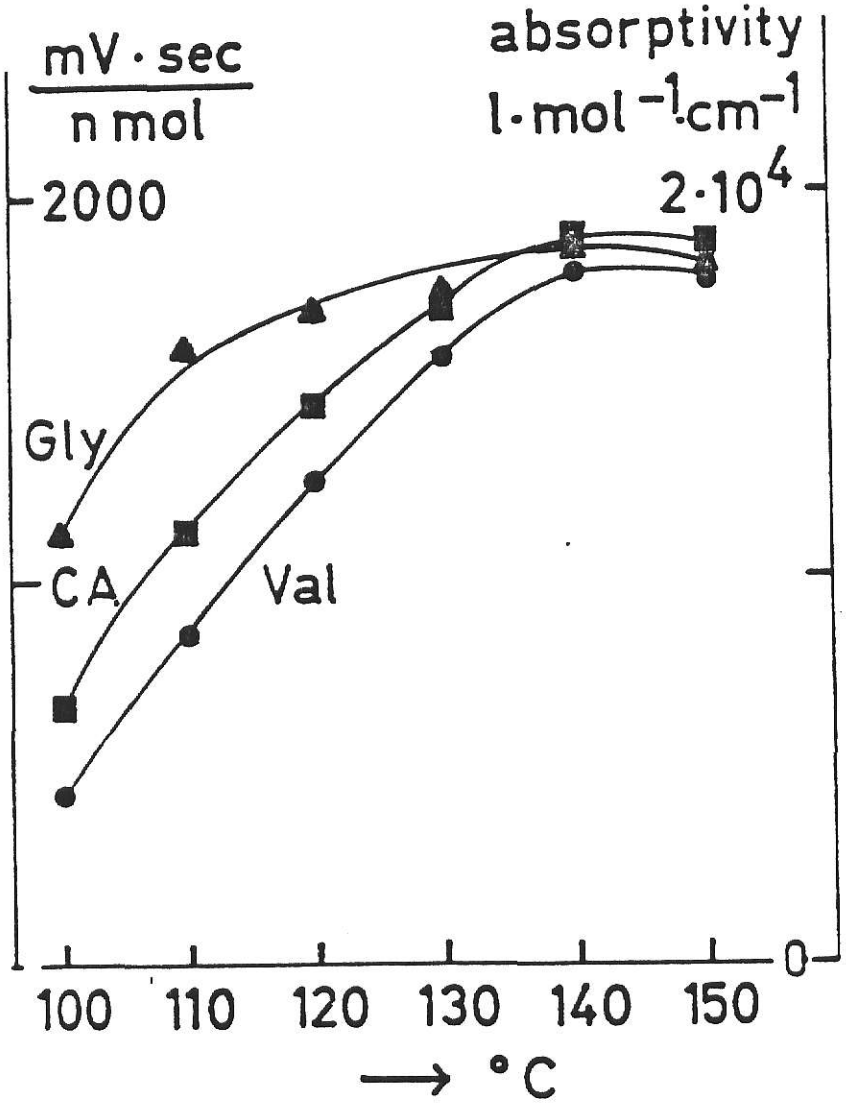

Fig. 2. Dependence of sensitivity on reactor temperature for reaction time of one minute (Jonker et al., 1978).

Hydrochloric acid is the most extensively used hydrolysis reagent. Moore and Stein (1963) describe its use with sealed pyrex tubes. A 5 mg sample of air-dried or lyophilized protein is weighed into a 16 x 125 mm heavy walled pyrex test tube. One mililiter of 6 N HCl is added to the tube. A section of the tube about 3 cm from the top is constricted with an oxygen flame to about a 1 mm bore. The lower half of the tube is inserted in a bath of solid carbon dioxide and ethanol to freeze the sample. When frozen, the tube is connected to a vacuum line and evacuated with an oil pump to 60 um. The tube is removed from the dry ice bath and the frozen solution is allowed to thaw slowly with the pump still on. As the bubbles form and rise in the tube, it is immersed momentarily in the dry ice bath to break the bubbles and allow the liquid to drain back. During this degassing process the pressure increases to about 80 um. When the pressure is back down to 60 um the tube is shaken to insure complete gas removal and then sealed. The hydrolysis is conducted at $110^{\circ}C \pm 1^{\circ}C$ for 20 or 70 hours. The temperature must be accurately controlled during hydrolysis. Following hydrolysis the tube is cooled to room temperature and centrifuged to remove any liquid from the walls. Then it is scored with a file at a point below the tapered end, cracked by the use of a hot glass rod and the cut end fire-polished. The hydrolysate is transfered to a round bottom flask and the HCl is removed under vacuum at $40^{\circ}C$ with a rotary evaporator. The sample residue is dissolved with water and taken to dryness again to insure complete removal of the HCl. The residue is transferred quantitatively to a 5 ml volumetric flask and brought to volume by the use of pH 2.2 buffer containing 5 ml per liter of thiodiglycol. The diluted sample is then filtered by vacuum filtration through Whatman No. 52 filter paper to remove insoluble humin. Aliquots of the filtrate

are then used for amino acid analysis (Thachuk and Irvine, 1969).

Another method of HCl hydrolysis refluxes the sample in excess HCl (Davies and Thomas, 1973). The sample (100 mg) is refluxed in a 150 ml flask with 100 ml 6 N HCl (distilled in an all glass apparatus). The flask is immersed in an oil bath to a depth where the liquid level is just above the oil level. Hydrolysis is conducted at 137°C ± 2°C and oxygen-free nitrogen is bubbled continuously through the liquid. Samples are hydrolyzed for 20 to 96 hours. After hydrolysis the samples are filtered through a G4 sintered glass filter and washed with 250 ml distilled water. The hydrolysate is made up to 500 ml. A rotary evaporator at 40°C is used to take a 25 ml aliquot of the hydrolysate to dryness. The residue is dissolved in 5 to 10 ml of 0.1 N HCl depending on the protein content of the sample.

During acid hydrolysis, threonine and serine are progressively destroyed (Rees, 1946). Their decomposition varies according to the protein being hydrolyzed and to unknown factors (Noltmann et al., 1962). As hydrolysis time is lengthened, aspartic acid, glutamic acid, proline, and arginine appear to be decomposed to a small but measurable degree and there is substantial decomposition of cystine and tyrosine (Hirs, Stein, and Moore, 1954). In the absence of air, tryptophan is relatively stable during acid hydrolysis except in the presence of serine, hydroxyproline, cystine, and carbohydrates (Gruen, 1973). Gruen believed the major factor in the extensive destruction of tryptophan is the degradative oxidation by cystine to cysteine. Oxidation during acid hydrolysis may lead to the formation of significant amounts of cysteic acid from cystine, methionine sulfoxide from methionine, and chlorotyrosine from tyrosine if the tubes are not evacuated below 100 um (Moore and Stein, 1963). Not all amino

acids undergo destruction. Smith et al., (1954) found alanine to be stable at both 20 and 70 hours of hydrolysis. Glycine, leucine, and phenylalanine appear to be stable for up to 140 hours of acid hydrolysis. Valine and isoleucine reach maximal values only after 70 hours of acid hydrolysis and remain constant up to 140 hours (Noltmann, et al., 1962).

Sulfonic acids are non-oxidizing strong acids and have been used to replace HCl in acid hydrolysis. Liu and Chang (1971) used p-toluenesulfonic acid (PTSA) as the catalyst for hydrolysis of proteins. Hydrolysis was carried out in heavy walled 18 x 150 mm ignition tubes. The protein (2 to 3 mg) was hydrolyzed under vacuum (20 to 30 um) at 110°C for 22, 48, and 72 hours with 1 ml of 3 N PTSA containing 0.2% 3-(2-amino ethyl)indole. After hydrolysis 2.0 ml of 1 N NaOH was added and the sample was transfered quantitatively to a 5.0 ml volumetric flask and brought to volume with water. After filtration through a 0.22 u millipore filter an aliquot was used for amino acid analysis. They noted that at 22 hours of hydrolysis 3 N PTSA had a lower yield of valine and isoleucine than 6 N HCl. After 48 and 72 hours the yields were quite similar for the two methods. Serine and threonine yields were somewhat higher with PTSA, but all the other amino acids, except tryptophan, gave comparable recoveries.

p-Toluenesulfonic acid can be used as a method of hydrolysis for tryptophan determination. Recovery of tryptophan is very high when purified proteins and PTSA are used. The results compare very well to those from methods of tryptophan determination that do not use acid hydrolysis. The addition of 0.2% 2-(2-amino ethyl)indole resulted in a higher and more reproducible recovery of tryptophan. However, an excess of carbohydrate in a sample causes extensive tryptophan destruction. The carbohydrate content is limited to 2.0 mg when 2 to 4 mg of protein are being hydrolyzed with

1.0 ml of acid.

A p-toluenesulfonic acid hydrolysis procedure is a more convenient method for routine amino acid determinations, because the hydrolysate can be placed directly on the column of the amino acid analyzer without prior removal of the solvent as required when HCl is used. A single hydrolysate can be used to determine all of the amino acids including tryptophan when the sample has a low carbohydrate content.

Davies and Thomas (1973) performed extensive hydrolysis studies on lysozyme, casein, and a microfungal protein comparing many hydrolysis reagents. They found lysozyme gave comparable values with HCl and PTSA, but casein and microfungal protein gave lower values for cystine and methionine when hydrolyzed with PTSA. Votisky and Gundel (1973) compared PTSA and HCl acid hydrolysates using fishmeal and grain maize. They reported PTSA to be a softer hydrolysis method giving lower yields than HCl. This study compares the recovery of amino acids from HCl and PTSA hydrolysates using a cereal product at twelve different time periods. From this a pattern of amino acid release and destruction can be followed for each amino acid and one can then compare equally PTSA and HCl hydrolysis.

MATERIALS AND METHODS

Comparison of HCl and PTSA Acid Hydrolysis

Approximately 100 mg of KSU flour were accurately weighed into each of 72 16 x 125 mm pyrex test tubes. Four ml of 6 N HCl were added to one half of the tubes and 4 ml of 3 N PTSA were added to the rest of the tubes. To all tubes 0.5 ml of 12.5 uM/ml norleucine, an internal standard, was added. Each tube was covered with a polypropylene culture tube cap (Bacti-Capalls, A. H. Thomas, Co.) and placed in a 100°C boiling water bath equipped with an air condenser to recover the water. The HCl and PTSA hydrolyses were conducted in separate reaction vessels. Time 0.0 hours began when the tubes were placed in the boiling baths. At 5, 10, 15, 20, 24, 30, 35, 40, 50, 60, 72, and 100 hours from time 0.0, three sample tubes were removed from both the PTSA and HCl hydrolysis baths to give three hydrolysates at each time for each method.

The PTSA samples were cooled and 1 ml of diluter buffer (Table 1) was added. The hydrolysate was transferred quantitatively to a 10 ml volumetric flask and brought to volume with deionized water. After cooling the HCl samples were transfered quantitatively to a 125 ml round-bottom flask with deionized water and dried with a rotary evaporator under vacuum at 40°C to remove the HCl. The samples were reconstituted with deionized water and taken to dryness a second time to insure complete removal of the HCl. One ml of diluter buffer was added to the dried sample before it was quantitatively transferred to a 10 ml volumetric flask and brought to volume with deionized water.

A 1 ml aliquot of each hydrolysate was placed in a 1.5 ml polypropylene microcentrifuge tube and centrifuged at 15500 rcf for 3 minutes to remove insoluble humin. The remaining hydrolysate was transferred to a 16 x 125 mm

Table 1

Buffers for Single Column Automatic Amino Acid Analysis

Buffer	pH	Ion	Sodium Conc. in M/L	# ml/L 88% Phenol
Diluter	2.20	Formate	2.0	0.0
A	3.25	Formate	0.2	1.0
B	4.25	Formate	0.2	1.0
C	6.80	Citrate	0.2	1.0
D	10.00	Borate	0.2	1.0
E	12.25	Borate	0.2	1.0

pyrex test tube, covered with parafilm and frozen. The clarified supernatant was loaded directly onto a Dionex D-300 kit or component analyzer (Sunnyvale, CA) by filling a 25 ul sample loop in a sixteen place automatic sampler. A standard containing 0.625 uM/ml of each amino acid and norleucine was analyzed after every seven samples. At injection the sample loop contents were pumped onto a single 0.4 x 15 cm stainless steel column of Durrum DC-5A spherical polysulfonated resin. Twenty minutes after injection the column temperature was changed from 45° to 65°C. The selection of buffers, column temperature, and sample injection were controlled by a Dionex CP-3 chromatographic programmer.

The buffer system (Table 1), a modification of Hare (1972), consisted of two sodium formate buffers (A and B), a sodium citrate buffer (C), a sodium borate buffer (D), and a sodium borate regeneration buffer (E). All buffers had a 0.2 M sodium concentration and were pumped at a flow rate of 18 ml/hr.

Detection of the amino acids was accomplished by pumping a ninhydrin reagent (2% ninhydrin, 0.400 g stannous chloride dihydrate in 875 ml of methyl cellosolve and 125 ml of 4 N pH 5.51 sodium acetate buffer) into the effluent stream at a rate of 18 ml/hr. The effluent-ninhydrin mixture was then carried through a boiling water bath in a coil of teflon tubing (0.3 mm i.d. and 18 feet long) to develop Rhuhemann's Purple. A photometer that alternately senses 570 nm, 440 nm, and a reference wavelength was used to detect the colored complex. Background interference was corrected by subtracting the absorbance at the reference wavelength from the absorbance at each analytical wavelength. An output for both the 570 nm and 440 nm wavelengths was charted on a chart recorder. Both channels were set at 1.0 on the photometer and the recorder full scale setting was 0.01 volts.

Peak areas were calculated by a Supergrator-3 programmable computing integrator (Columbia Scientific Industries). Integrator output was printed on paper tape and recorded by a Techtran 817A data cassette recorder. Recorded data were entered through a Perkin Elmer CRT terminal into an IBM 370 computer and calculated. Data from each hydrolysate was calculated in units of grams of amino acid per 100 grams of protein. The triplicate values for each hydrolysis time and each amino acid were averaged and a plot was made of time versus percent amino acid. The HCl and PTSA plots for each amino acid were graphed together for comparison. The data was also normalized by correcting to a 100% recovery protein basis.

Sensitivity

A standard containing 0.625 uM/ml of each amino acid and norleucine was analyzed by a Dionex automatic amino acid analyzer as described in the hydrolysis methods section. Flow rates of the effluent and the ninhydrin reagent were 18 ml/hr and 9 ml/hr respectively. The ninhydrin formulation consisted of 2% ninhydrin, 0.400 g stannous chloride dihydrate in 750 ml methyl cellosolve and 250 ml 4 N pH 5.51 sodium acetate buffer (Spackman et al., 1958). A reaction coil containing 18 feet of teflon tubing (0.3 mm i.d.) was used in a boiling water bath. Water in the reaction bath was then replaced by 85% acetoin (3-hydroxy 2-butanone). Acetoin has a boiling point of 148°C, is nontoxic and exhibits a pleasing aroma. The 85% acetoin boiled at 108°C and a standard was analyzed at this temperature. The areas of each amino acid at each temperature were compared.

After distillation to remove water the acetoin was placed in the reaction bath and brought to a boil (temperature = 138°C). A 20 ft length of teflon tubing (0.3 mm i.d.) was connected to the exit port of the flow

cell to increase back pressure to prevent boiling of the eluent-ninhydrin mixture in the coil. A standard was analyzed at this temperature but the run was not completed due to problems that developed from the 138°C temperature.

Water was added to the acetoin to lower the boiling point to 128°C. A standard was analyzed using an 18 ft coil in the 128°C acetoin bath. The coil was shortened repeatedly by 3 ft lengths and a standard was analyzed at each length. The peak areas of the 6, 9, 12, 15, and 18 ft coils were then compared to a standard analyzed using an 18 ft coil in a 100°C water bath. All analysies were done using a ninhydrin flow rate of 18 ml/hr and a eluent flow rate of 18 ml/hr. The photometer was set at 2.0 for both channels and the recorder full scale setting was 0.01 volts. The ninhydrin formulation contained 2% ninhydrin, 0.400 g stannous chloride dihydrate in 875 ml of methyl cellosolve and 125 ml 4.0 M pH 5.51 sodium acetate.

An 18 ft coil in a 100°C bath and a 6 ft coil in a 128°C bath were used to analyze 144 picomoles of each amino acid plus norleucine. All analyzer conditions were the same as described in the previous paragraph except the photometer setting was changed to 0.1 for both channels. The chromatograms and peak areas were compared.

Isatin

A set of eight 16 x 125 mm pyrex test tubes containing 0.1 ml of 12.5 uM/ml proline, 2.4 ml of deionized water, and 2.5 ml of 2% isatin were prepared to determine the optimum reaction time of isatin with proline. The tubes were placed in a boiling water bath and one tube was removed every 30 seconds. After cooling the absorbance of each tube was read at 570 nM

on a Beckman DB-G grating spectrophotometer. A comparison was made of reaction time versus absorbance.

The amount of isatin needed for optimum color development was found by pipetting 0.1 ml of 12.5 uM/ml proline into five 16 x 125 mm pyrex test tubes. To this 0.5, 1.0, 1.5, 2.0, or 2.5 ml of 2% isatin in methyl cellosolve was added. The mixture was brought to a 5 ml total volume with deionized water. The tubes were stirred with a vortex mixer and heated for 3 minutes in a boiling water bath. After cooling the absorbance was read at 570 nM and the number of ml of isatin was compared to absorbance.

The absorbance maximum and the pH optimum was then determined. Buffer solutions of 0.1 M were prepared for pH 2, 3, 4, and 5 using potassium acid phthalate (KHP), pH 6 and 7 using sodium dihydrogen phosphate, and pH 8 and 9 using trihydroxyaminomethane (TRIS). A trial was conducted at each pH by the following procedure:

Tube #1 - 3 ml buffer plus 3 ml 2% isatin in methyl cellosolve (Blank).

Tube #2 - 2.45 ml buffer plus 0.05 ml 12.5 uM/ml proline and 2.5 ml 2% isatin in methyl cellosolve.

Both tubes were mixed on a vortex stirrer and placed in a boiling water bath for three minutes, cooled, and scanned on a Beckman DB-G grating spectrophotometer from 750 to 320 nM. Both blank versus blank, and blank versus sample were scanned for each pH.

The exact pH optimum for the isatin-proline complex was found by utilizing a series of 0.1 M KHP buffers with a pH ranging from 2.5 to 4.0 with 0.3 gradations. Each tube contained 2.5 ml buffer, 2.5 ml 2% isatin in methyl cellosolve, and 0.05 ml of 12.5 uM/ml proline. Absorbance at 595 nM was read after mixing and heating for 3 minutes, and cooling. A plot of absorbance of absorbance versus pH was made.

The color yield of lysine and ammonia with isatin was examined. Three